Heligmosomoides polygyrus inhibits established colitis in IL-10-deficient mice

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Inflammatory bowel disease (IBD) is prevalent in industrialized countries, but rare in less-developed countries. Helminths, common in less-developed countries, may induce immunoregulatory circuits protective against IBD. IL-10$^{-/-}$ mice given piroxicam develop severe and persistent colitis. Lamina propria mononuclear cells from colitic IL-10$^{-/-}$ mice released IFN-γ and IL-12. The ongoing piroxicam-induced colitis could be partially blocked with anti-IL-12 monoclonal antibody suggesting that the inflammation was at least partly IL-12 dependent. Colonization of piroxicam-treated colitic IL-10$^{-/-}$ mice with *Heligmosomoides polygyrus* (an intestinal helminth) suppressed established inflammation and inhibited mucosal IL-12 and IFN-γ production. *H. polygyrus* augmented mucosal IL-13, but not IL-4 or IL-5 production. Transfer of mesenteric lymph node (MLN) T cells from IL-10$^{-/-}$ animals harboring *H. polygyrus* into colitic IL-10$^{-/-}$ recipients inhibited colitis. MLN T cells from worm-free mice did not. *Foxp3* (scurfin) drives regulatory T cell function. *H. polygyrus* enhanced *Foxp3* mRNA expression in MLN T cells that had regulatory activity. This suggests that *H. polygyrus* inhibits ongoing IL-10$^{-/-}$ colitis in part through blocking mucosal Th1 cytokine production. Resolution of inflammation is associated with increased IL-13 production and can be adoptively transferred by MLN T cells.

Received	17/12/03
Revised	7/7/04
Accepted	23/7/04

Key words: Helminth / Cytokines / IL-12 / Autoimmunity / Mucosa / T lymphocytes

1 Introduction

Although there is a genetic predisposition to Crohn's disease (CD), epidemiological evidence suggests a strong environmental contribution to the risk of developing inflammatory bowel disease (IBD). The incidence of CD rose dramatically within one generation during the 1940–1980s [1]. CD is common in developed countries with temperate climates where the rates of colonization with helminthic parasites rapidly declined after the 1940s [2].

Helminths alter their host's immune responses [3], which may help limit development of potentially pathological-type inflammations [2, 4]. Earlier animal studies suggested that helminths protect mice from IBD. Mice rectally exposed to trinitrobenzene sulfonic acid (TNBS) develop colitis that is prevented by inhibiting Th1 cytokines [5]. Previously, we showed that mice systemically exposed to eggs of the helminthic parasite *Schistosoma mansoni* are resistant to TNBS-induced colitis [6]. This resistance to TNBS colitis requires IL-4 and intact Stat 6 circuitry, and is associated with depressed IFN-γ and augmented IL-10 colonic mRNA levels [6].

IL-10 is an important immunoregulatory cytokine that down-modulates macrophage activation and accessory cell function [7]. Mice rendered IL-10 deficient by gene disruption (IL-10$^{-/-}$) develop chronic colitis, influenced by colonic flora [8]. The colitis is characterized by infiltration of the lamina propria (LP) with CD4$^+$ T cells and augmented IFN-γ production [9]. The onset of the intestinal inflammation is delayed by treatment with anti-IFN-γ or anti-IL-12 mAb, demonstrating that the colitis requires an exuberant Th1 response. This excellent model of colitis suffers from variability in onset and extent of disease among individual mice. The spontaneous intestinal inflammation takes more than 3 months to develop in specific pathogen-free (SPF) facilities. How-

[DOI 10.1002/eji.200324833]

Abbreviations: CD: Crohn's disease **Cox:** Cyclooxygenase **LP:** Lamina propria **MC:** Mononuclear cells **IBD:** Inflammatory bowel disease **MLN:** Mesenteric lymph node **TI:** Terminal ileum

ever, treatment with piroxicam, a nonsteroidal anti-inflammatory drug (NSAID), initiates uniformly severe chronic colitis in IL-$10^{-/-}$ mice [10].

Piroxicam inhibits both cyclooxygenase 1 (Cox1) and 2 (Cox2), impeding prostaglandin production. Low doses of piroxicam, as used here, do not induce inflammation in normal mice, but precipitate intense colitis in IL-$10^{-/-}$ mice within 7 days [10]. The colitis persists after withdrawal of piroxicam and is characterized by infiltration of the LP with IFN-γ-producing $CD4^+$ T cells [10]. Piroxicam treatment of IL-$10^{-/-}$ mice provides a uniform, chronic colitis that permits evaluation of immunomodulatory therapies.

Heligmosomoides polygyrus is an intestinal helminth that colonizes mice. Unlike *S. mansoni*, *H. polygyrus* inhabits only the duodenal mucosa. Mice become colonized by ingesting larvae that briefly invade the duodenal wall. The larvae then re-enter the lumen and mature into adult worms. Mice colonized with *H. polygyrus* have a blunted delayed-type hypersensitivity response to ovalbumin [11]. Colonization with *H. polygyrus* induces immunosuppressive peritoneal macrophages that have an activated phenotype [12].

Using the piroxicam-synchronized IL-$10^{-/-}$ model of chronic IBD, the objective of this study was to determine if an intestinal helminth that colonizes only the duodenum of the host could reverse ongoing Th1-type colitis. It was discovered that *H. polygyrus* reversed previously established, ongoing chronic intestinal inflammation and inhibited mucosal Th1 cytokine production in IL-$10^{-/-}$ colitis. Adoptive transfer of mesenteric lymph node (MLN) T cells from worm-colonized IL-$10^{-/-}$ colitis-free donors into IL-$10^{-/-}$ colitic recipients also reverses the inflammation, suggesting that the protection was T cell dependent.

2 Results

2.1 Effect of *H. polygyrus* on Th1 intestinal inflammation

Five-week-old IL-$10^{-/-}$ mice were given NSAID (piroxicam) for 2 weeks. Previous studies showed that this treatment induces severe Th1-type colitis in IL-$10^{-/-}$ mice by the end of the 2-week exposure. Moreover, the inflammation persists after stopping the piroxicam [10]. As expected, IL-$10^{-/-}$ mice given piroxicam developed severe inflammation of the colon and to a lesser extent of the terminal ileum upon stopping the drug. This inflammation remained active in mice evaluated 16 days after stopping piroxicam. There was intense lymphocytic infiltration of the LP, which also extended deep into the bowel wall and out to the serosa. Focal mucosal ulcerations, crypt abscesses and hypertrophy of the epithelial and muscle layers were observed (Fig. 1A–C).

Mice were colonized with *H. polygyrus* 2 days after stopping piroxicam, and their colons were examined

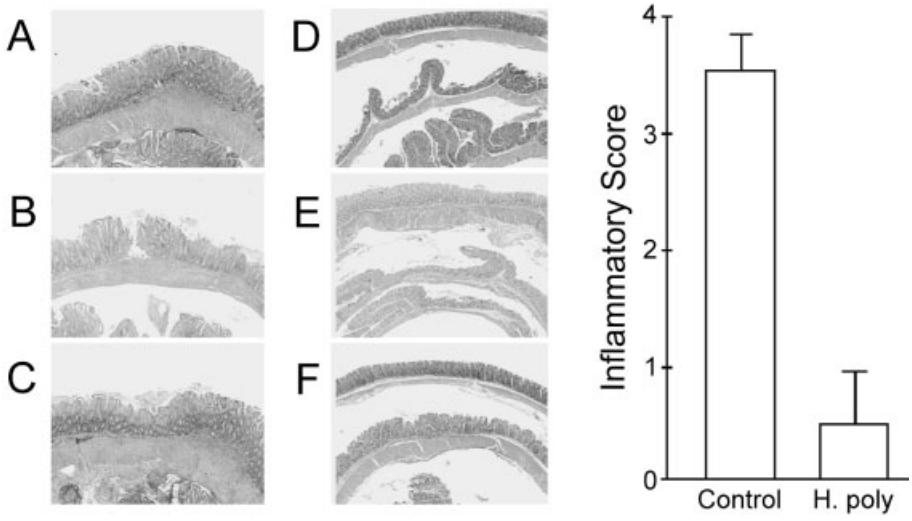

Fig. 1. Effect of *H. polygyrus* colonization on established colitis. (A–C) Typical colonic inflammation 2 weeks after finishing piroxicam treatment in IL-$10^{-/-}$ mice that did not receive *H. polygyrus*. (D–F) Typical colon histology in mice colonized with *H. polygyrus* for 2 weeks after piroxicam treatment. A–F Hematoxylin and eosin staining, $\times 40$. Bar graph shows colitis inflammatory scores. Inflammation was scored on a 0–4 scale by pathologists blinded to the treatment group. Data are means $\pm$SE from 11 animals studied in two separate experiments.

14 days later. Unlike the control group, these colons showed a minimal lymphocytic infiltration restricted to the LP, and there was no muscle or epithelial cell hypertrophy (Fig. 1D, E). The histological score fell from 3.6±0.4 (mean ± SE) in the controls to 0.55±0.5 in colonized mice (p=0.001).

H. polygyrus remained viable within the duodenum throughout the course of the experiment. Visual and histological evaluation of the duodenal mucosal revealed no evidence of inflammation or tissue damage.

2.2 Effect of *H. polygyrus* on mucosal IFN-γ and IL-12p40 production

Next, we examined the affect of *H. polygyrus* on mucosal Th1 cytokine secretion. IL-10$^{-/-}$ mice were treated with piroxicam, and LP mononuclear cells (LPMC) were isolated 16 days after stopping the piroxicam treatment. LPMC from mice without *H. polygyrus* released IFN-γ (Fig. 2A) and IL-12p40 (Fig. 2B) when cultured *in vitro*. IL-12p40 was only detected in cell culture supernatants after CpG oligonucleotide stimulation. IL-12 p70 was not detected. However, no IFN-γ was detected in the culture supernatants of LPMC isolated from mice bearing *H. polygyrus*, even after anti-CD3/CD28 stimulation ($p<0.001$). IL-12p40 production was substantially decreased ($p<0.05$).

Blockade of IL-12p40 partially improves established spontaneous colitis in IL-10$^{-/-}$ mice [13]. Mice with piroxicam-initiated colitis were treated with anti-IL-12 mAb to determine if blocking this cytokine would reverse established colitis in this model. IL-10$^{-/-}$ mice were treated with piroxicam to induce colitis, and were then given blocking anti-IL-12p40 mAb (C17.8). Mice that received blocking anti-IL-12 mAb had diminished colitis (2.11±0.14) compared to controls (3.58±0.11, $p<0.05$). Unstimulated LPMC isolated from anti-IL-12-treated mice constitutively released less IFN-γ (0.1±0.06 ng/ml) than LPMC from control mice (1.6±0.1 ng/ml, $p<0.001$). Anti-CD3/CD28-stimulated LPMC from anti-IL-12-treated mice produced 1.6±0.1 ng IFN-γ/ml, while those from control mice made 9.6±0.8 ng IFN-γ/ml ($p<0.001$). Data are from two experiments with six mice per group.

2.3 Effect of *H. polygyrus* on mucosal Th2 cytokine production

We also studied the capacity of LPMC to make IL-4 and IL-5. Intestinal LPMC isolated from IL-10$^{-/-}$ mice 16 days after piroxicam administration produced little or no IL-4

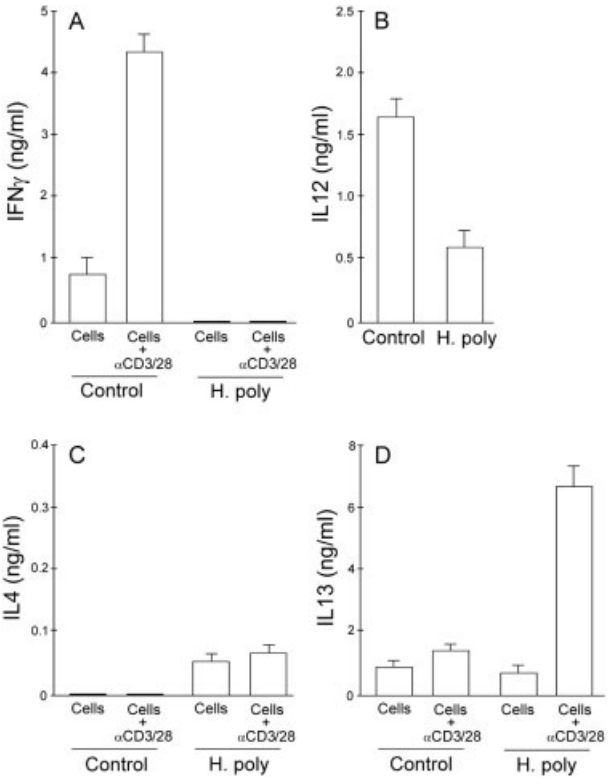

Fig. 2. Effect of *H. polygyrus* colonization on mucosal IFN-γ, IL-12, IL-4, and IL-13 production. LPMC were isolated from piroxicam-treated mice that did not receive *H. polygyrus* (Control) or from mice treated with piroxicam then colonized with *H. polygyrus* (H. poly). LPMC were cultured in the absence or presence of anti-CD3 and anti-CD28 (αCD3/28) and supernatants assayed for IFN-γ (A), IL-4 (C), or IL-13 (D). LPMC also were stimulated with CpG and supernatants assayed for IL-12p40 (B). Data are means ± SE from at least three independent experiments.

(Fig. 2C). *H. polygyrus* colonization minimally enhanced the constitutive secretion of IL-4 from LPMC. T cell activation with anti-CD3/CD28 stimulation did not affect IL-4 secretion. There was no IL-5 detected in any LPMC culture supernatants even after *H. polygyrus* colonization or T cell stimulation.

IL-13 is a Th2 cytokine that shares some of the functions of IL-4. LPMC from IL-10$^{-/-}$ mice with colitis secreted small amounts of IL-13 constitutively. LPMC from mice bearing *H. polygyrus* produced similar amounts. However, T cell activation with anti-CD3/CD28 substantially increased IL-13 secretion only from LPMC derived from the mice colonized with *H. polygyrus* (Fig. 2D, $p<0.005$).

TGF-β is a regulatory cytokine that can inhibit inflammation and IFN-γ release. We tested for TGF-β production by LPMC cultured in reduced-serum conditions. We

found that constitutive or anti-CD3/CD28-stimulated production of TGF-β by LPMC from *H. polygyrus* colonized mice was similar to that of control mice (data not shown).

2.4 Flow analysis of dispersed LPMC populations

Isolated, dispersed LPMC were examined for T cell composition using flow cytometry. LPMC from piroxicam-treated mice with or without *H. polygyrus* contained $CD4^+$, $Thy1.2^+$ T cells in similar proportions (Table 1). The LPMC from *H. polygyrus* colonized mice had a reduced $CD8^+$ T cell compartment. This difference was not present in MLN cells. LPMC and MLN cells from both groups contained similar proportions of $CD4^+$ $CD25^+$ T cells.

2.5 Adoptive transfer of MLN cells to colitic mice

While *H. polygyrus* did not injure the intestinal mucosa in the IL-$10^{-/-}$ piroxicam-treated mice, it did induce hypertrophy of MLN, which were about three times normal size. We then investigated whether cells from these hypertrophic MLN could substitute for *H. polygyrus* and suppress piroxicam-synchronized, intestinal inflammation.

Table 1. Percentage T cell distribution in LPMC and MLN of piroxicam-treated IL-$10^{-/-}$ mice with and without helminth colonization[a)]

	Control	*H. polygyrus*
LPMC		
$Thy1.2^+$, $CD4^+$	22.9±1.5	27.6±3.0
$Thy1.2^+$, $CD8^+$	16.6±1.9	6.6±0.8*
$CD4^+$, $CD25^+$	2.6±0.5	3.1±0.4
MLN		
$Thy1.2^+$, $CD4^+$	21.2±4.1	27.6±3.0
$Thy1.2^+$, $CD8^+$	16.2±0.7	21.9±3.8
$CD4^+$, $CD25^+$	2.8±0.1	3.1±0.1

[a)] Percentage of $CD4^+$, $CD8^+$ or $CD4^+$ $CD25^+$ T cells in dispersed LPMC or MLN from IL-$10^{-/-}$ mice 2 weeks following piroxicam treatment as determined by flow cytometry. Control mice were not colonized with *H. polygyrus*. Other mice were colonized with *H. polygyrus* after piroxicam treatment was discontinued. Results are given as mean % ± SD determined by two or three independent experiments. *Control *vs. H. polygyrus*, $p<0.05$.

IL-$10^{-/-}$ mice were treated with piroxicam to induce colitis. Two days after stopping piroxicam, some of the mice received MLN cells from healthy IL-$10^{-/-}$ mice, while others were transferred the same number of cells from IL-$10^{-/-}$ mice bearing *H. polygyrus*. The colons from the cell recipients were examined 14 days later (Fig. 3A). The IL-$10^{-/-}$ mice receiving MLN cells from healthy IL-$10^{-/-}$ donors maintained severe colitis (score =3.4±0.15). However, recipients of MLN cells from animals harboring *H. polygyrus* had minimal intestinal inflammation (score =0.4±0.17, $p<0.001$). Flow analysis of dispersed MLN cells from mice colonized with *H. polygyrus* contained $CD4^+$ and $CD8^+$ T cells in proportions similar to the MLN cells of uninfected animals (Table 1). Thus, recipients of MLN cells received similar numbers of T cells.

Next, we investigated whether transfer of MLN T cells was sufficient to reverse colitis. In experiments conducted as outlined above, IL-$10^{-/-}$ mice continued to have colitis after receiving MLN T cells from mice without *H. polygyrus* (score =3.3±0.2, Fig. 3B). Mice that received MLN T cells from *H. polygyrus*-colonized mice had improved colitis (score =1.3±0.3, $p=0.005$).

Colonization with *H. polygyrus* induced MLN T cells that had regulatory activity. *Foxp3* (scurfin) is a transcription factor required for regulatory T cell function. We examined if *H. polygyrus* exposure altered *Foxp3* mRNA expression by MLN T cells using real-time PCR [14]. Colonization with *H. polygyrus* induces a 3.2-fold increase in *Fox3p* mRNA expression ($p=0.02$) by MLN

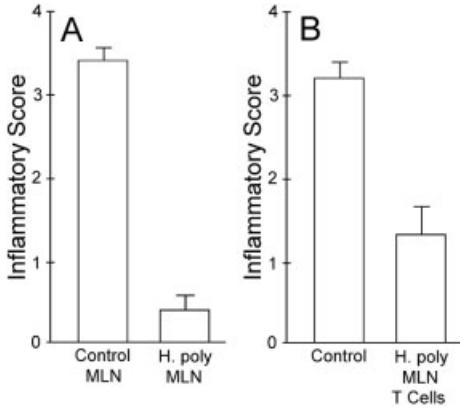

Fig. 3. Effect of MLN cell transfer on established colitis. Piroxicam-treated colitic mice received dispersed MLN cells from IL-$10^{-/-}$ animals without *H. polygyrus* colonization (Control) or MLN cells from mice colonized with *H. polygyrus*. (A) Mice received 2×10^7 unfractionated MLN cells. (B) Mice received 5×10^6 MLN T cells isolated by negative selection. Data are means ± SE from at least nine animals/group studied in two separate experiments.

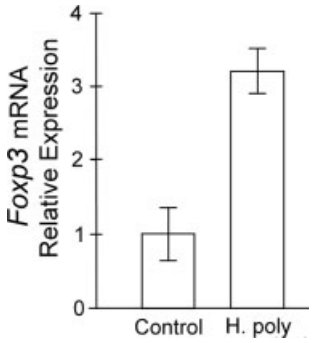

Fig. 4. Effect of *H. polygyrus* colonization on MLN T cell *FoxP3* mRNA expression. RNA was isolated from dispersed MLN (as in Fig. 3), reverse transcribed, and analyzed for content of *Foxp3* mRNA normalized to HPRT by real-time PCR. Results are from five separate experiments with six mice per group.

T cells as compared to non-colonized mice (Fig. 4). Isolated MLN cells from control or colonized mice that were depleted of T cells did not express *Foxp3* mRNA (data not shown).

3 Discussion

The chronic intestinal inflammation that characterizes CD results from a dysregulated immune response to luminal contents. The dysregulated response is associated with elevated IFN-γ [15, 16] and phosphorylated Stat 1 [17] expression. Murine models of CD, like the IL-10 deficient mouse, develop colitis that expresses a similar cytokine pattern.

Individual IL-10$^{-/-}$ mice in the same colony and even the same cage develop spontaneous colitis at a non-uniform rate. This variability in disease onset makes it difficult to use IL-10$^{-/-}$ mice as a model for disease treatment. However, exposing IL-10$^{-/-}$ mice to piroxicam uniformly initiates chronic colitis that persists after the drug is withdrawn [10]. This piroxicam-synchronized colitis shares the cytokine profile of spontaneous colitis and is similar to that seen in CD [10]. Thus, the piroxicam-synchronized IL-10$^{-/-}$ colitis is an excellent model to dissect immunoregulatory effects of therapeutic interventions. We used the piroxicam-synchronized colitis model to determine if colonization with an intestinal helminthic parasite affects previously established chronic Th1-type intestinal inflammation.

Mice treated with piroxicam developed severe, persistent colitis characterized by transmural lymphocytic inflammation, mucus depletion, crypt abscesses, and frequent ulceration. Remarkably, colonization with *H. polygyrus* reversed this severe inflammation after it was established. This dramatic reduction in previously established colitis occurred in the absence of immunoregulatory IL-10.

Colitis in the IL-10$^{-/-}$ mouse is associated with increases in Th1 cytokine production [9]. Onset of spontaneous inflammation is inhibited by blocking either IFN-γ or IL-12 [13]. We examined LPMC cytokine production to see if colonization with *H. polygyrus* influenced mucosal Th1 circuitry.

We found that colonization with *H. polygyrus* dramatically curtailed IFN-γ secretion. The IFN-γ ELISA used was sensitive to 30 pg/ml. *H. polygyrus* caused a greater than 99% inhibition of mucosal IFN-γ expression. Colonization also produced a threefold reduction in LPMC production of IL-12. These reductions were not due simply to lack of colitis. Cultured LPMC isolated from worm-free IL-10$^{-/-}$ mice without colitis produced easily detectable IFN-γ. Therefore, colonization with *H. polygyrus* appears to directly and dramatically inhibit LPMC Th1 cytokine production.

Established IL-10$^{-/-}$ colitis is not abrogated by blocking IFN-γ, although blocking IL-12 does reduce established inflammation [13]. We studied piroxicam-synchronized colitis to determine if IL-12p40 blockade reverses inflammation in this model. Similar to previous observations [13], IL-12p40 blockade did improve colitis and inhibited LPMC IFN-γ production. Treatment with anti-IL-12p40 mAb has also been shown to inhibit established colitis in wild-type mice with TNBS-induced inflammation [5] or in the CD45RB^{hi} *Scid* transfer IBD model [18]. A pathological dysregulated immune response is responsible for colitis in each of these models.

The blocking anti-IL-12p40 antibody also inhibits activity of IL-23. IL-23 is a heterodimer of IL-12p40 and p19 rather than p35 present in IL-12 [19]. IL-23 promotes proliferation and IFN-γ production by memory T cells. Transcription of mRNA for the p19 component is up-regulated in LP dendritic cells from mice with Rag$^{-/-}$ CD4^{+} $\alpha\beta$T cell transfer colitis [20]. It is possible that the reduction in IL-12p40 that occurs with *H. polygyrus* colonization may reflect decreased IL-23 release. This also is inferred by the absence of measurable p70 in the cell culture supernatants.

One of the anti-colitic mechanisms induced by *H. polygyrus* colonization may be inhibition of LPMC IL-12 or IL-23 production. Yet, treatment with anti-IL-12p40 mAb did not resolve colitis to the extent achieved by colonization with *H. polygyrus.* This may be due to insufficient blockade by anti-IL-12p40. Alternatively, it is

likely that *H. polygyrus* colonization induces additional anti-inflammatory circuits to inhibit colitis.

In wild-type mice, *H. polygyrus* induces a strong Th2 response that helps limit colonization [21, 22]. Strong Th2 responses can inhibit development of excessive Th1 responses [23, 24]. For example, prior exposure to Th2-inducing eggs of *S. mansoni* inhibits development of TNBS colitis through a mechanism that requires intact IL-4 and Stat6 signaling [6].

We examined the effect of *H. polygyrus* colonization on IL-4 production by LPMC from previously colitic IL-$10^{-/-}$ mice. Unlike wild-type mice, previously colitic IL-$10^{-/-}$ mice did not respond to *H. polygyrus* with an increase in T cell-stimulated, IL-4 production. Thus, it is unlikely that *H. polygyrus* colonization reverses IL-$10^{-/-}$-associated colitis by augmenting T cell IL-4 release. However, constitutive production of IL-4, from an LPMC source not stimulated by anti-CD3/CD28, was slightly augmented in *H. polygyrus*-colonized mice and, thus, may have contributed to the anti-inflammatory response.

In contrast to IL-4, LPMC from worm-colonized IL-$10^{-/-}$ mice stimulated with anti-CD3/CD28 produced sevenfold more IL-13 than LPMC from worm-free mice. IL-13 inhibits pro-inflammatory TNF-α, IL-1, and NO release from activated macrophages [25, 26]. IL-13 treatment inhibits onset of diabetes in NOD mice [25]. Virally transduced intra-articular expression of IL-13 inhibits adjuvant-induced arthritis in rats [27]. It is possible that *H. polygyrus*-induced augmentation of mucosal T cell IL-13 production helped to reverse the established colitis.

H. polygyrus resides in the duodenum. The helminth does not inhabit the colon or ileum, but as shown in this study it suppresses established colitis. This suggests that *H. polygyrus* induces regulatory-type cells that circulate to the colon and inhibit the intestinal inflammation. We tested for the presence of cell-mediated regulation by transferring MLN cells from *H. polygyrus*-colonized IL-$10^{-/-}$ mice into IL-$10^{-/-}$ mice with established piroxicam-synchronized colitis. Unlike MLN cells from worm-free IL-$10^{-/-}$ mice, MLN from colonized mice abrogated established colitis. Again, this suppression occurred in the absence of IL-10-mediated immunoregulation. MLN T cells isolated from colonized mice also adoptively transferred suppression, indicating that *H. polygyrus* induced a population of T cells with regulatory activity that are normally absent in the MLN of IL-$10^{-/-}$ mice.

Scurfin is a transcription factor encoded by *foxp3* that controls regulatory T cell development and activity [28, 29]. Colonization of IL-$10^{-/-}$ mice with *H. polygyrus* significantly increased MLN T cell *Foxp3* mRNA expression. This supports our conclusion that *H. polygyrus* exposure activates regulatory T cells.

Several types of regulatory T cells have been described [30]. Induction and function of Tr1 cells requires IL-10 that is absent in this system. Therefore, it is unlikely that MLN contain classical Tr1 cells, which reverse colitis. Further characterization of the regulatory-type IL-$10^{-/-}$ T cells induced by *H. polygyrus* is ongoing.

In summary, we show for the first time that colonization with an intestinal helminth can inhibit an established IL-12-dependent colitis and reverse intestinal pathology. Colonization with *H. polygyrus* inhibits IFN-γ and IL-12 release, but promotes T cell-stimulated, IL-13 production by LPMC. Furthermore, a helminth that is limited to the duodenal mucosa induces T cells within the MLN compartment that can suppress colitis upon adoptive transfer. These observations support the hypothesis that the dramatic rise in autoimmune disease present in developed countries may result at least in part from the eradication of helminthic parasites [2].

4 Materials and methods

4.1 Mice and induction of colitis

This study used wild-type and IL-$10^{-/-}$ C57BL/6 mice. Breeding colonies for the animals were maintained in SPF facilities at the University of Iowa. To induce colitis, 5-week-old IL- $10^{-/-}$ mice were given piroxicam (Sigma, St. Louis, MO) mixed into their feed (NIH-31 M) for 2 weeks. They received 60 mg piroxicam/250 g food during week 1 and 80 mg piroxicam/250 g food during week 2. Mice subsequently were placed on the normal rodent chow without piroxicam. The colitis was evaluated 16 days after stopping piroxicam.

In some experiments, mice were colonized with *H. polygyrus*, beginning 2 days after stopping the piroxicam. Colonization was initiated with 200 third stage larvae (L3) of *H. polygyrus* given by gastric gavage. Infective, ensheathed *H. polygyrus* L3 (U.S. National Helminthological Collection no. 81930) were obtained (from fecal cultures of eggs) by the modified Baermann method and stored at 4°C until used.

4.2 Histological analysis of colitis

Colons (from the ileocecal value to the mid descending colon) were opened longitudinally and rolled up onto a glass rod. The tissue was fixed in 4% neutral buffered formalin, removed from the glass rods without unrolling the tissue and processed for sectioning. Tissue was sliced to obtain

longitudinal sections of colon that were 6 μm thick, and then stained with hematoxylin and eosin for light microscopic examination. The inflammation was scored as 0–4 using the following criteria: 0, no change from normal tissue; 1, patchy MC infiltrates in the LP; 2, more uniform MC inflammation involving both the epithelium and LP (accompanied by minimal epithelial hyperplasia and slight to no depletion of mucous from goblet cells); 3, some epithelial and muscle hypertrophy with patchy lymphocytic infiltrates extending into the muscle layers (with mucus depletion and occasional crypt abscesses and epithelial erosions); 4, lesions involved most of the intestinal section. The inflammation in the latter category was comprised mostly of lymphocytes and some neutrophils, and was transmural and severe. There was prominent thickening of both the epithelial and muscle layers. There was mucus depletion and more frequent crypt abscesses. Ulcerations were frequent.

4.3 Cell isolation and T cell enrichment

Gut LPMC were isolated as described below. Intestinal tissue was washed extensively with RPMI, and all visible Peyer's patches were removed with scissors. The intestine was opened longitudinally, cut into 5-mm pieces and then incubated in 0.5 mM EDTA in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) for 20 min at 37°C with shaking to release intraepithelial lymphocytes and epithelial cells. This was repeated after thorough washing. Tissue was then incubated 20 min at 37°C in 20 ml RPMI containing 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 5 mg/ml gentamycin, and 100 mg/ml streptomycin (all GIBCO) as well as 1 mg/ml collagenase (Sigma, CO130). At the end of the incubation, the tissue was subjected to further mechanical disruption using a 1-ml syringe. To remove debris, the LPMC preparations were washed through a pre-wetted gauze, layered in a funnel, with RPMI. LPMC were then washed once and sieved through a pre-wetted 2-cm nylon wool column gently packed into a 10-ml syringe. After washing, cells (up to 2×10^7) were layered onto a 30:70%-gradient Percoll column. Cells were spun at 2,200×g at room temperature for 20 min. The LPMC collected from the 30:70 interface were washed and maintained on ice until used. Cell viability was 90% as determined by eosin Y exclusion.

Single-cell suspensions of MLN were prepared by gentle teasing in RPMI 1640 medium (GIBCO, Grand Island, NY). The MLN cells were washed three times in RPMI. For cell transfer experiments, MLN T cells were isolated by negative selection using the SpinSep enrichment procedure employing antibody-coated, dense particles as described by the manufacturer (#17031, Stem Cell Technologies, Vancouver, Canada). Flow cytometry was used after each separation to assure appropriate recovery and purity (>98%) of the Thy^+ T cells.

4.4 Adoptive cell transfer

MLN cells were isolated from IL-$10^{-/-}$ mice colonized 2 weeks with *H. polygyrus* or from age-matched healthy IL-$10^{-/-}$ mice without such colonization. These unfractionated, dispersed MLN cells (2×10^7cells/mouse) or MLN T cells (5×10^6 cells/mouse) then were transferred into colitic IL-$10^{-/-}$ mice 2 days after discontinuation of piroxicam treatment by i.p. injection. The colitis was evaluated 14 days later.

4.5 Cell culture

For cytokine analysis, cells were cultured for 48 h in 96-well microtiter plates (Corning, Cambridge, MA) with 200 μl medium (5×10^5 cells/well) at 37°C. The culture medium was RPMI containing 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 5 mg/ml gentamycin, and 100 mg/ml streptomycin (all GIBCO). For most experiments, the cells were cultured alone or with anti-CD3 (2C11, ATCC) and anti-CD28 mAb (PharMingen, San Diego, CA) (each at 1 μg/ml). Isolated T cells were cultured in wells previously coated overnight with anti-CD3 and anti-CD28 mAb. For IL-12 analysis, cells were cultured with the synthetic phosphorothioate backbone oligonucleotide ODN 1826 (TCCATGACGTTCCTGACGTT) that contains two (underlined) immunostimulatory CpG motifs [31, 32], provided by the Coley Pharmaceutical Group (Wellesley, MA), and used at 0.6 μg/ml to stimulate production.

4.6 Flow cytometric analysis

MLN cells or LPMC were washed twice and adjusted to 10^7 cells/ml in FACS buffer (HBSS containing 1% FCS and.02% sodium azide). The cell suspensions then were dispensed into microcentrifuge tubes each containing 10^6 cells in 100 μl FACS buffer and stained with saturating amounts of conjugated antibodies for 30 min at 4°C. Following staining, cells were washed twice and re-suspended for analysis on a Becton Dickinson FACS 440 flow cytometer (Becton Dickinson, Mountain View, CA).

Before adding labeled mAb, each tube received 1 μg 2.4G2 antibody (anti-FcγR; ATCC) to block nonspecific binding of conjugated antibodies to Fc receptors. The other mAb used for staining were anti-CD4-Cy5 (RM2511; CalTag, Burlingame, CA), anti-CD8a-PE (53–6.7; Sigma), anti-Thy 1.2-FITC (TS; Sigma), anti-CD19-FITC (1D3; PharMingen) and anti-CD25-PE (PC61; PharMingen).

4.7 ELISA

ELISA was used to measure the concentrations of various cytokines in the supernatants. To measure IFN-γ, plates were

coated with an mAb to IFN-γ (HB170, ATCC) and incubated with supernatant. IFN-γ was detected with polyclonal rabbit anti-IFN-γ (gift from Dr. Mary Wilson, University of Iowa) followed by biotinylated goat anti-rabbit IgG (Accurate Chemical Co., Westbury, NY). Color development used streptavidin-horseradish peroxidase (Zymed San Francisco, CA) and TMB substrate (Endogen, Woburn, MA), and plates were read at 490 nm. IL-4 was captured with 11B11 (HB191, DNAX Research Institute, Palo Alto, CA) and detected with biotinylated BVD6 (provided by Kevin Moore and John Abrams, DNAX). IL-5 was captured with TRFK5 and detected with biotinylated TRFK4 (Dr. Robert Coffman, DNAX). Total IL-12 (p70 and p40) used anti-IL-12 mAb (Endogen) to capture p70 + p40 (Wistar Institute, Philadelphia, PA). Detection used biotinylated and affinity-purified C17-8 (Endogen). IL-13 was measured using goat anti-murine IL-13 (AF-413-NA, R&D Systems, Minneapolis, MN) for capture and biotinylated rat anti-mouse IL-13 mAb (MAB413, R&D Systems) for detection.

Active TGF-β was measured using acid-treated supernatant and MAB240 for capture and biotinylated BAF240 for detection (both from R&D Systems). Sensitivity of the ELISA was 30 pg/ml for IFN-γ, IL-4 and IL-5. mAb to IFN-γ, IL-4, IL-5 and IL-12 were from cell lines maintained in our laboratory. These mAb were purified from culture supernatants by ammonium sulfate precipitation.

4.8 Treatment of mice with anti-IL-12 mAb

In some experiments, mice were given 1 mg anti-IL-12p40 mAb (C.17.8 [33]) i.p. on days 1 and 7 after stopping piroxicam. Controls received vehicle (PBS). Colitis was evaluated 14 days after stopping piroxicam.

4.9 Measurement of *Foxp3* transcripts

Total RNA was extracted from freshly isolated MLN cells [34] and reverse transcribed with Moloney-monkey leukemia virus (400 U) using oligo-dT as primer. For real-time PCR analysis each three concentrations of each sample (0.2, 0.04, and 0.008 μg RNA) were amplified for *Foxp3* using 5′-CCCAGGAAAGACAGCAACCTT-3′ and 5′-TTCTCACAAC-CAGGCCACTTG-3′ for primers and 5′-FAM-ATCCTACC-CACTGCTGGCAAATGGAGTC-3′ as probe [14]. The results were normalized to HPRT amplified using 5′-TGAAGAGC-TACTGTAATGATCAGTCAAC-3′ and 5′-GCAAGCTTG-CAACCTTAACCAT-3′ as primers and 5′-JOE-TGCTTTCCCTGGTTAAGCAGTACAGCCC-3′ as probe. Amplification was performed using Taqman universal master mix (Applied BioSystems) and analyzed with an ABI PRISM 7700 Sequence Detection System.

4.10 Statistical analysis

Data are means ± SE of multiple determinations. Difference between two groups was compared using Student's *t*-test. *p* values <0.05 were considered significant.

Acknowledgements: Grants from the National Institutes of Health (DK38327, DK58755, DK02428, DK25295, AI49382), the Crohn's and Colitis Foundation of America, Inc. and the Veterans Administration supported this research.

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